[Supplement Material: EHP ms 09-01633-ART]

Bone lead measurements

Bone lead levels were measured using K-shell X-ray fluorescence (KXRF) instrument (ABIOMED, Danvers, MA, USA). A technical description and validity specifications of this instrument have been published elsewhere (Aro et al. 2000; Burger et al. 1990; Hu et al. 1990) In 1999, we replaced our prototype ABIOMED instrument with an upgraded instrument designed to improve measurement precision, with changes in the cadmium radiation source, adjustments to the geometry of the measurement procedure, and upgrades in both the software and specific hardware components of the system.(Aro et al. 1994) Briefly, 30-minute measurement was taken at the mid-shaft of the left tibia and at the left patella after each region was washed with a 50% solution of isopropyl alcohol. The KXRF beam collimator was sited perpendicular to the bone surface, providing unbiased point estimate of bone lead levels that is normalized for bone mineral content as micrograms of lead per gram bone mineral. The instrument also provides an estimate of the uncertainty associated with each measurement. Lead estimates with uncertainty values > 10 μg/g for tibia and > 15 μg/g for patella were excluded as unreliable, a standard protocol in analyses of bone lead.

HFE genotyping

We genotyped the participants for both the *C282Y* and *H63D* polymorphisms of the *HFE* gene (GenBank accession no. Z92910; http://www.ncbi.nlm. nih.gov/sites/entrez) as previously described (Feder et al. 1998; Park et al. 2006; Wright et al. 2004). The genomic DNA was extracted from the archived blood with commercially available PureGene Kits (Gentra Systems, Minneapolis MN). Multiplex PCR assays were designed using Sequenom SpectroDESIGNER software by inputting sequence containing the SNP site and 100 bp of flanking sequence on either side of the SNP. For this assay, 4 SNPs were multiplexed. HFE RS1800562 & RS1799945, Transferrin RS1049296 and ALAD RS1800435. For this study only the results for

the HFE RS1800562 & RS1799945 SNPs were analyzed. The SNPs were grouped into multiplexes that 1) all the use the same mix of terminating nucleotides, 2) that are most likely to produce a PCR product in multiplex, and 3) that will produce an extension product over the SNP site that does not overlap in mass with any other oligonucleotide present in the reaction mix. The PCR was carried out in 384-well reaction plates in a volume of 5 ul using 2.5 ng genomic DNA. Multiplex PCR was carried out to generate short PCR products (> 100 bp) containing one SNP or insertion deletion. Briefly, 2.5 ng genomic DNA was amplified in a 5 ul reaction containing 1 x HotStar Taq PCR buffer (Qiagen), 2.5 mM $MgCl_2$, 200 uM each dNTP, 50 nM each PCR primer, 0.1 U HotStar Taq (Qiagen). The reaction was incubated at 95^{o}C for 15 minutes followed by 45 cycles of 95^{o}C for 20 seconds, 56^{o}C for 30 seconds, 72^{o}C for 1 minute, followed by 3 minutes at 72^{o}C. Excess dNTPs were then removed from the reaction by incubation with 0.3 U shrimp alkaline phosphatase (USB) at 37^{o}C for 20 minutes followed by 5 minutes at 85^{o}C to deactivate the enzyme. Single primer extension over the SNP or insertion/deletion was carried out in a final concentration of 600 nM each extension primer, 50 uM d/ddNTP and 0.126 U Thermosequenase (Solis Biodyne) and incubated at 94^{o}C for 2 minutes followed by 45 cycles of 94 ^{o}C for 5 seconds, 52 ^{o}C for 5 seconds, and 72 ^{o}C for 5 seconds. The reaction was then desalted by addition of a cation exchange resin followed by mixing and centrifugation to settle the contents of the tube. The extension product was then spotted onto a 384 well spectroCHIP before being flown in the MALDI-TOF mass spectrometer. Specifically, the following primers were used in the multiplex assay.

ALAD RS1800435-

Forward PCR primer 5'-ACGTTGGATGTTCAACCCCTCTACCCACAC-3'

Reverse PCR primer	5'-ACGTTGGATGAGATCAAGACACAGCGTAGG-3'
Extension primer	5'CTCAGCATCTCTTCCAGCCG-3'

Transferrin RS1049296

Forward PCR primer	5'-ACGTTGGATGTGAGTTGCTGTGCCTTGATG-3'
Reverse PCR primer	5'-ACGTTGGATGATCTTTCCGTGTGACCACAG-3'
Extension primer	5'-CGCATACTCCTCCACAG-3'

HFE RS1799945

Forward PCR primer	5'-ACGTTGGATGTCTACTGGAAACCCATGGAG-3'
Reverse PCR primer	5'-ACGTTGGATGTTGAAGCTTTGGGCTACGTG-3'
Extension primer	5'-GCTGTTCGTGTTCTATGAT-3'

HFE RS1800562

Forward PCR primer	5'-ACGTTGGATGTACCCCAGATCACAATGAGG-3'
Reverse PCR primer	5'-ACGTTGGATGTGGATAACCTTGGCTGTACC-3'
Extension primer	5'-GAAGAGCAGAGATATACGT-3'

As a quality control measure, 10% of DNA samples from the participates were randomly selected and run in duplicate. In parallel to genotyping our study population, the control blood with known *HFE* mutation status (the wild-type genotype and heterozygous and homozygous for both *H63D* and *C282Y* variants) were also examined for validation propose.

Supplemental Material, Table 1. Characteristics of men at the baseline lead measurements by *HFE* genotyping[*].

Characteristic	*HFE* identified	Missing *HFE* Data
No.	(N=632)	(N= 112)
Continuous variables (mean ±SD)		
Age	66.5±6.9	70.0±7.5[§]
Alcohol(g/day)	13.6±18.3	11.2±17.7
BMI	27.8±3.6	27.9±4.7
Calories (kcal)	1976±634	1987±672
Calcium(mg)	797±372	873±485
Potassium(mg)	3299±1073	3991±2060
Sodium(mg)	3810±1712	3647±1561
Heart rate (beats/min)	73.0±10.3	75.5±12.16
Heamotocrit	43.9 ±3.2	43.2 ±3.0
Hemoglobin (g/dl)	15.1± 1.2	14.9± 1.1[§]
Fasting glucose (mg/dl)	108.6± 28.2	119.5± 48.4
Height(m)	1.73±0.07	1.72±0.06
LDL[†] (mg/dl)	111.9±37.1	109.6±38.6
HDL[‡](mg/dl)	48.4±12.8	47.6±11.7
Total/HDL Ratio	5±1.45	5±1.32
Smoking (pack/years)	21.2±25.2	25.6±24.5
Waist circum.(cm)	98.1±9.3	98.9±11.4
SBP (mm/Hg)	135.4±17.6	135.4±15.9
DBP (mm/Hg)	81.6±9.6	79.9±9.7
PP (mm/Hg)	53.8±14.5	55.5±12.1
Categorical variable [n (%)]		
Education		

Unfinished high school	67(10.6)	12(11.8) [#]
High school	377(59.7)	49(48.5)
College and above	185(29.3)	40(39.6)
Family history of HTN	238(37.6)	36(34.2)
Hypertension	255(40.3)	45(40.8)
Ischemic heart diseases	102(16.1)	20(17.9)
Diabetes	39(6.1)	14(12.5) [§]
Hypertension treatment	54(8.5)	19(16.9) [§]
hyperlipidemia treatment	71(11.2)	8(7.1)
Diabetes treatment	28(34.4)	10(8.9)
Bone Lead levels [median (IQR)]		
Tibia lead (μg/g)	19 (13–27)	22(15.5–29) [§]
Patella Lead(μg/g)	26 (18–37)	32(22–44) [§]

[*] Values are Mean ± SD or %; [†] LDL = low density lipoprotein; [‡] HDL = high density lipoprotein; [§] *P*-value <0.05; [#] Missing education data in 11 excluded subjects.

Reference

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